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RESEARCH ARTICLE

Rapid Plant Regeneration, Analysis of Genetic Fidelity and Camptothecin Content of Micropropagated Plants of *Ophiorrhiza mungos* Linn. – A Potent Anticancer Plant

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Abstract

An efficient protocol has been established for rapid multiplication of *Ophiorrhiza mungos* Linn. (Rubiaceae), a potent anticancer plant. Axillary and terminal buds of these *in vitro*-raised seedlings formed the primary source of explants for direct organogenesis. Explants were inoculated onto MS medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BA) and kinetin (KN). The best morphogenic response was observed on MS media supplemented with 0.25 mg L^{-1} BA and 0.25 mg L^{-1} KN, which exhibited the highest regeneration frequency (84%), the maximum number of shoots/explants (63.1 ± 1.35) and shoot length (2.8 ± 1.15) within 4 weeks. Fortification of 1.0 mg L^{-1} GA_3 enhanced the shoot elongation by 2.33 fold in 91% of shoot cluster cultures within 3 weeks. A high percent frequency of rooting (92.13%) was achieved within 15 days of shoot implantation on ½ strength MS media fortified with 100 mg L^{-1} activated charcoal. The rooted plantlets were successfully acclimatized with 95% survival rate. Randomly amplified polymorphic DNA (RAPD) analysis confirmed that all the regenerated plants were genetically identical to their mother plant, suggesting an absence of detectable genetic variation in the regenerated plantlets. High performance liquid chromatography (HPLC) was done to further confirm the existence of qualitative and quantitative differences in the major secondary metabolite (camptothecin) between the mother plant and *in vitro*-propagated plants. The present results evidently showed comparable chemical profiles. Thus, the present protocol can be used for clonal mass propagation of true-to-type elite *O. mungos* plants.

Key words : campothecin, genetic fidelity, HPLC, *in vitro* propagation, *Ophiorrhiza mungos*, RAPD.

Abbreviations

BA: Benzyl-6-adenine, KN: kinetin, MS: Murashige and Skoog, NAA: α-Naphthalene acetic acid, IAA: Indole-3-acetic acid, GA_3: Gibberellic acid, CTAB: Cetyltrimethyl ammonium bromide, PCR: Plolymerase chain re-action, HPLC: High performance liquid chromatography

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Introduction

Camptothecin (CPT), a monoterpene indole alkaloid originally identified in *Camptotheca acuminata,* is one of the effective anticancer drugs (Wall et al. 1966). According to Redinbo et al. (1998), CPT and their derivatives exert their action by inhibiting DNA topoisomerase I. CPT is very active against parasitic trypanosomes, leishmania, falciparum malaria, and microorganisms (Bodley et al. 1998; Nandhakumar et al. 2002). CPT is also known to occur in many plant species like *Merriliodendron megacarpum, Nothapodytes nimmoniana,* and *Eravatamia heyneana.* Among these, *Ophiorrhiza* species belonging to the family Rubiaceae such as *O. mungos, O. Pumila*, *O. rugosa, O. liukiuensis, O kuroiwai, O. alata,* and *O. prostata* are novel sources for this most valuable compound (Saito et al. 2001; Watase et al. 2004). An approximate estimation on global turnover for CPT and its derivatives in 2004 was around one thousand million US dollars which is equal to one tonne of CPT in terms of raw materials (Lorence et al. 2004; Watase et al. 2004).

With an increasing demand for CPT and its analogues to treat cancers, there has been an indiscriminate felling of trees yielding CPT (Romanelli et al. 1998). Though Nothapodytes and Camptotheca species have been reported to have a higher CPT content than Ophiorrhiza, it is to be noted that the herb species can be easily cultivated within a short span of time. There is a limited distribution of this plant in some areas of Eastern and Western Ghats. The rate of plant propagation is critical to meet the commercial requirement of camptothecin. A slow propagation rate in *O. mungos,* because of low seed viability and germination rate as well as a small number of propagules (stem cuttings), has restricted the natural dissemination of the plant. Also, the destruction caused by harvesting the roots as a source of the drug has threatened the survival of the plant. Plant tissue culture can be the best approach to solve this problem. Cell and tissue culture studies of several CPT producing from Ophiorrhiza species have been investigated as alternative sources for CPT (Beegum et al. 2007; Martin 2007; Roja 2008; Saito et al. 2001; Vineesh et al. 2007; Watase et al. 2004). However, *in vitro* regeneration studies of *O. mungos* are very scanty (Jose and Satheesh Kumar 2004; Namdeo et al. 2012). In these studies, fruits and seedlings were used for *in vitro* propagation and the source of plant was restricted to Western Ghats of India, Kerala, India. However, Ophiorrhiza species are distributed throughout the Western Ghats of India. Also, there is no confirmation on the genetic reliability and authentication of plantlets quality derived from their *in vitro* propagation studies.

In clonal propagation studies, the genetic fidelity of regenerants can be increased by using explants with preformed meristems. In general, to improve shoot proliferation from explants, the medium is added with plant growth regulators. On the other hand, this increases the possibility of cellular mutations and the genetic variability in the regenerated plants (Bairu et al. 2006; Howell et al. 2003) and can cause somaclonal variations (Larkin and Scowcroft 1981). Therefore, it is required to assess the genetic fidelity of the regenerants by using cytological or molecular markers (Mallon et al. 2010). There are many techniques such as cytological, isozymes, and molecular markers to detect variation and confirm the true-to-type nature of the *in vitro*-derived plantlets. However, RAPD markers are widely employed to evaluate genetic stability and quality among the clonally propagated plants and mother plant, thus ensuring the quality of tissue cultured plantlets (Chalageri and Babu 2012; Paul et al. 2010; Sun et al. 2009).

Hence, this study was undertaken to establish an efficient plant propagation protocol through direct organogenesis from axillary and terminal buds of *O. mungos*. This is probably the first report on tissue culture studies of wild *O. mungos* plant species collected from Western Ghats of India, Karnataka, India. The genetic fidelity and chemical profiles of micropropagated plants were verified by RAPD and HPLC analysis, respectively, to confirm the true-to-type nature of the plantlets.

Materials and Methods

Collection of plant material and explants preparation

Mother plants of *O. mungos* were collected from a wild population from Western Ghats of Kemmannugundi hills, Chickmagalore district of Karnataka, India in the month of October and authenticated from Department of Botany, Sri Krishnadevaraya University, Anantapur, India. The voucher specimen (ROM/10/10) was maintained at Rishi Foundation, Bangalore, India. The collected plants were established in the greenhouse of Rishi Foundation, Bangalore, India. The explants (axillary and terminal buds) were collected from healthy growing plants and dissected under aseptic conditions to initiate shoot multiplication.

Media and culture conditions for shoot multiplication

Under aseptic conditions, the explants were inoculated on MS (Murashige and Skoog 1962) medium, containing 2% (w/v) sucrose, supplemented with different concentrations and combinations of BA (0.25, 0.5, 1.0, and 2.0 mg L^{-1}) and KN (0.25, 0.5, 1.0, and 2.0 mg L^{-1}) for shoot proliferation and multiplication. A control without any plant growth regulator was included. The pH of the medium was adjusted to 5.8 prior to the addition of 0.8% agar and autoclaved at 121°C, 15 lbs. pressure for 15 min. All the cultures were incubated at 25 ± 2°C under a 16 h light and 8 h dark regime with a light intensity of 3,000 lux provided by cool-white fluorescent tubes. Data with respect to percent shoot multiplication, number of shoots/explants, and shoot length per culture were recorded after 30 days of subculture. Each treatment, including the control had a total of 20 explants.

Shoot elongation

Cultures were incubated under the same growth conditions as stated above. The explants with shoot clusters, produced after 4 weeks of culture on MS medium containing 0.25 mg L^{-1} BA and 0.25 mg L^{-1} KN, were transferred to MS medium supplemented with varied concentrations of gibberellic acid (GA_3) (0.25, 0.5, 1.0, 1.5, and 2.0 mg L^{-1}) for 3 weeks to allow for the elongation of shoots. The mother explants were repeatedly subcultured on shoot multiplication medium after each harvest of the elongated shoots.

In vitro rooting and acclimatization

After multiplication, regenerated shoots were separated and transferred to the rooting medium. The regenerated shoots (5 - 6 cm) bearing at least 4 - 5 internodes were excised and cultured on freshly prepared rooting medium containing half-strength or full-strength MS medium supplemented with different concentrations of activated charcoal (100 and 200 mg L^{-1}), IAA and NAA (0.5 and 1.0 mg L^{-1}). After 4 weeks of culture, the frequency of root formation, number of roots produced per cultured shoot, and length of the root were recorded. Plantlets with well-formed roots were removed from culture medium, washed gently in running tap water, and transferred to net pots containing sterile soil, sand, and manure (1 : 1 : 1). These plantlets were covered with a polythene cover ensuring high humidity (80%) and watered for every 3 - 4 days with quarter-strength MS salt solution without sucrose and vitamins. After 20 days, the polythene cover was removed and maintained in net pots, and subsequently transferred to the field.

Molecular characterization of regenerated plants

DNA was isolated from leaves of randomly selected hardened *in vitro* plants after 3 months of their transfer to field conditions and mother plants. DNA was also extracted from the *in vivo* mother plant. DNA was isolated from 100 mg regenerated leaf samples of field-grown plants (control) using the modified cetyltrimethyl ammonium bromide (CTAB) method outlined by Doyle and Doyle (1987). The quality of the extracted DNA was verified by sample electrophoresis on 0.8% (w/v) agarose gels prepared in 1X TAE buffer. Aliquots of 100 bp uncut lambda DNA ladder (100 ng μL^{-1}) were used as standards. Ethidium bromide stain (1 mg L^{-1}) was used to visualize the DNA. The concentration of DNA was measured by spectrophotometric analysis.

PCR amplification was carried out in 25 µL volume using five different decamer primers (Operon Technologies Inc., USA) that were selected based on available literature (Jaimsha Rani 2012). The reaction mixture consisted of 1U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), 0.25 µL each of dNTP (10 mM), 5 pmol of decamer primer, 1X polymerase buffer (2.5 µL), 0.25 mM $MgCl_2$, and 50 ng of DNA sample. The amplification was performed using a thermal cycler (Eppendorf, Germany). The program consisted of initial denaturation at 94°C for 2 min, PCRs were run for 40 cycles consisting of a denaturation step of 1 min at 94°C, annealing for 1 min at 37°C, and an extension at 72°C for 2 min. A final extension was followed at 72°C for 5 min. The amplified products were checked in a 1.5% agarose gel stained with 0.5 µg mL^{-1} of ethidium bromide and documented by a gel documentation system (Vilber Lourmat, France).

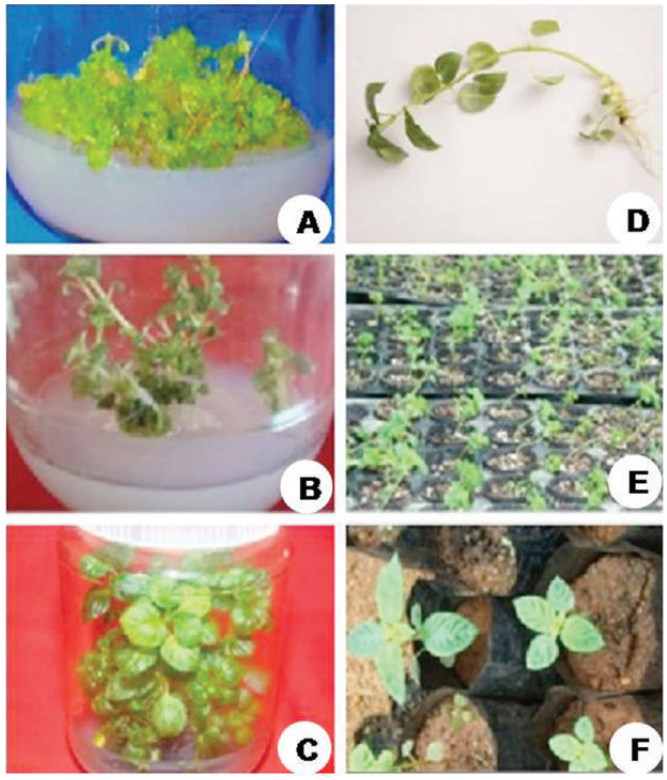

Fig. 1. Micropropagation of *Ophiorrhiza mungos*. (A, B) Initiation and multiple shoot regeneration from nodal segments on MS + 0.5 mg L^{-1} BA and 0.5 mg L^{-1} KN. (C) Prolific elongation of shoots from primary culture of *O. mungos* within 2 weeks of culture on MS and 1.0 mg L^{-1} GA_3. (D) A rooted shoot of *O. mungoson* MS (½) + activated charcoal (100 mg L^{-1}). (E) *In vitro*-raised plantlets transplanted to soil rite in net pots. (F) Directly acclimatized plantlet in the soil.

HPLC profiling and campothecin content

Mother plant material and tissue culture grown plant (whole plant) material of *O. mungos* were collected, washed, and dried at 55°C in an air drier. Dried biomass was defatted with petroleum ether and filtered. The residual mass was then extracted repeatedly with methanol five times and evaporated to dryness in a rotoflash evaporator and methanolic extract was dissolved in water. Aqueous methanolic extract thus obtained was then repeatedly extracted with chloroform 4 - 5 times. The combined chloroform extract was evaporated to dryness using an rotoflash evaporator. Quantitative HPLC was done out on water associate using Novapak C18 column (150 nm) and solvent system KH_2PO_4 : CH_3CN : MeOH (7 : 2 : 1) was used. The flow rate was adjusted to 1.2 mL min^{-1} and the detector was set at 254 nm. The concentrations of CPT was measured by standard calibration curves of authentic samples.

Statistical analysis

The experiments were set up in completely randomized

Table 1. Effect of different concentrations and combinations of BA and KN on regeneration frequency (%), number of shoots/explant, and shoot length (cm) from axillary and terminal buds of *O. mungos* cultured on MS medium after 30 days

Cytokinins (mg L^{-1})		Regeneration frequency (%)	umber of shoots/explants ± SD[1]	Shoot length (cm) ± SD[1]	Callus Formation[2]
BA	KN				
0.00	0.00	30	9.0 ± 0.50	0.3 ± 0.24	–
0.25	0.00	68	42.0 ± 1.20	1.7 ± 0.49	–
0.50	0.00	65	46.1 ± 0.83	2.2 ± 1.23	–
1.00	0.00	45	41.2 ± 1.65	1.2 ± 1.15	+
2.00	0.00	23	12.2 ± 1.86	0.6 ± 1.41	+
0.00	0.25	66	17.5 ± 0.96	1.7 ± 0.86	–
0.00	0.50	68	35.4 ± 0.88	2.4 ± 0.51	–
0.00	1.00	55	22.3 ± 1.90	2.2 ± 0.15	–
0.00	2.00	35	12.2 ± 1.16	1.5 ± 0.75	+
0.25	0.25	84	63.1 ± 1.35	2.8 ± 1.15	–
0.25	0.50	74	31.9 ± 1.67	2.6 ± 1.06	–
0.25	1.00	72	24.7 ± 1.29	2.3 ± 0.15	–
0.25	2.00	34	10.0 ± 1.23	1.6 ± 1.21	+
0.50	0.25	72	55.0 ± 0.48	2.5 ± 0.98	–
0.50	0.50	78	48.2 ± 0.75	2.6 ± 0.29	–
0.50	1.00	62	37.4 ± 1.40	2.3 ± 1.26	+
0.50	2.00	18	7.3 ± 1.53	1.2 ± 1.09	+
1.00	0.25	66	13.6 ± 0.23	1.9 ± 1.00	–
1.00	0.50	56	12.3 ± 0.60	2.3 ± 0.92	–
1.00	1.00	57	10.5 ± 0.58	1.9 ± 1.38	+
1.00	2.00	0	0.0 ± 0.00	0.0 ± 0.00	+
2.00	0.25	45	13.6 ± 0.31	1.6 ± 0.99	+
2.00	0.50	41	12.3 ± 1.60	0.8 ± 1.42	+
2.00	1.00	34	10.5 ± 1.31	0.4 ± 1.30	+
2.00	2.00	0	0.0 ± 0.00	0.0 ± 0.00	+
F-value[3]			*	*	
CD at 5% level			2.17	0.26	

[1] Data indicate mean ± standard deviation. Ten replicates were used per treatments and experiment was repeated thrice.

[2] +: Callus induction, –: No callus

[3] *: Significant at 5% level.

design. The different treatments were replicated thrice. Experimental data was recorded after 30 days of culture and subjected to Fisher's method of analysis of variance. Wherever, the 'F' test was significant for comparison of treatment means, C. D value was worked out at a probability level of 5%.

Results and Discussion

Shoot multiplication

The effects of different cytokinin types and concentrations on morphogenetic response of explants after 30 days of culture were explored and summarized in Table 1. Direct organogenesis was exhibited by explants on MS medium containing different concentrations of BA and KN (0.25, 0.5, and 1.0 mg L^{-1}) separately or in combinations. Shoot regeneration frequency ranging from 0 to 84% was obtained in all the treatments. All cytokinin concentrations generally increased shoot production when compared to the control. MS medium supplemented with 0.5 mg L^{-1} BA was best suitable for bud break and resulted in a maximum number of shoots/explant (46.1 ± 0.83); higher shoot length (2.2 ± 1.23 cm), and 65% of regeneration frequency (Fig. 1A). Among different concentrations of cytokinins tested, explants cultured on BA-supplemented media exhibited a superior response when compared to explants cultured on KN-fortified media. Similarly, Beegum et al. (2007) has reported that BA is the most effective cytokinin for induction of shoots in *Ophiorrhiza prostata*. The hormone BA is known to stimulate multiple shoots in *Passiflora mollussima* (Johnson et al. 2007), *Mentha viridis* (David and Arockiasamy 2008), and *Rubia* cardifolia (Swaroopa Ghatge et al. 2011). The concentration of cytokinin used in the culture medium plays a significant role in shoot organogenesis (Sun et al. 2009; Kumaraswamy and Anuradha (2010). According to Roja (2008), the medium containing 4 mg L^{-1} BA and 0.5 mg L^{-1} NAA was effective in regeneration of shoot cultures in *O. rugosa.* This is contradictory to our findings that increased concentrations of cytokinins beyond 0.5 mg L^{-1}, exhibited a decreased number of shoot buds coupled with callus proliferation. This may be due to faster cell division leading to profuse callus proliferation and resulting in the hindrance of morphogenesis. Similar results were obtained by Chaudhari et al. (2004) in *Tylophora indica*, Ahmad et al. (2008) in *Vitex negundo,* Nikam et al. (2009) in *Momordica cymbalaria,* Sudipta et al. (2011) in *Leptadenia reticulata*, Paul et al. (2010) and Swamy et al. (2014) in *Pogostemon cablin*. *O. mungos* is a very sensitive plant and it expresses its morphogenetic potentiality even at very low concentrations of cytokinins. Though KN is less effective on multiple shoot regeneration, it played a role in increasing the length and strength of shoots (Table 1). On KN-supplemented media, the regenerated shoots produced roots as well. The result is in agreement with the observations made by Beegum et al. (2007) in *O. prostrata*. Though KN was inferior for shoot proliferation in *O. mungos*, its usefulness either alone or with BA induced direct shoot organogenesis in *Asparagus maritimus* (Stajner et al. 2002) and *Bixa orellana* (De Pairva et al. 2003).

The combination treatment of BA and KN improved the frequency of multiple shoot formation, shoot number, and shoot length (Table 1). The best morphogenic response was found on MS media containing 0.25 mg L^{-1} BA and 0.25 mg L^{-1} KN, which exhibited the highest regeneration frequency (84%) (Fig. 1B). The maximum number of shoots/explants (63.1 ± 1.35) and shoot length (2.8 ± 1.15) was also evidenced on the same treatment. The regenerated shoots developed fragile roots following further culture. Enhancement in the induction of shoots by BA and KN in combination may be due to the synergy of cytokinins as reported in *Rollinia mucosa*, *Solanum surrattense* and *Pogostemon cablin* (Figueiredo 2001; Pawar 2002; Swamy et al. 2010). In *O. rugosa*, BA (4 mg L^{-1}) and NAA (0.05 mg L^{-1}) was used for shoot cultures (Roja 2008). Namdeo et al. (2012) used picloram, thidiazuron, and GA_3 in a ratio of 1 : 2 : 1 for shoot pro-

Table 2. Effect of gibberellic acid (GA_3) on elongation of shoots from nodal explants of *O. mungos* on MS medium supplemented with 0.25 mg L^{-1} BA and KN after 3 weeks of culture

GA_3 (mg L^{-1})	Shoot elongation response (%)	Mean shoot length (cm) ± SD[1]	Fold increase in shoot length ± SD
0.00	10	2.8 ± 0.12	0.00 ± 0.00
0.25	48	3.5 ± 1.46	0.72 ± 2.05
0.50	69	4.2 ± 1.01	1.41 ± 0.46
1.00	91	5.1 ± 1.35	2.33 ± 0.22
1.50	73	3.9 ± 1.41	1.15 ± 0.03
2.00	66	3.0 ± 0.17	0.24 ± 1.36
F-value[2]		*	*
CD at 5% level		0.81	1.01

[1] Data indicate mean ± standard deviation. Ten replicates were used per treatments and experiment was repeated thrice.

[2] *: Significant at 5% level.

Table 3. Effect of various concentrations of IAA, NAA, and Activated charcoal on rooting of proliferated shoots of *O. mungos* cultured on MS medium after 30 days

Medium (strength) + Auxin (mg L^{-1})	Root in duction (%) ± SD[1]	Number of roots/shoot (cm) ± SD[1]	Root length (cm) ± SD[1]
MS (½)	83.11	18.30 ± 1.02	5.11 ± 2.10
MS	81.22	15.27 ± 1.21	5.23 ± 0.41
MS (½) + IAA (0.5)	76.21	17.10 ± 1.10	5.67 ± 1.67
MS (½) + IAA (1.0)	69.53	21.66 ± 0.51	5.52 ± 2.02
MS (½) + NAA (0.5)	64.63	19.00 ± 0.09	4.87 ± 2.01
MS (½) + NAA (1.0)	56.10	20.78 ± 1.02	4.64 ± 1.29
MS (½) + Activated charcoal (100)	92.13	28.21 ± 1.14	6.30 ± 1.74
MS (½) + Activated charcoal (200)	91.86	25.80 ± 0.44	6.10 ± 0.53
MS + IAA (0.5)	56.10	14.76 ± 0.73	5.73 ± 0.33
MS + IAA (1.0)	65.16	16.22 ± 1.30	5.33 ± 1.34
MS + NAA (0.5)	69.43	15.07 ± 1.30	4.85 ± 0.24
MS + NAA (1.0)	54.73	15.02 ± 1.82	5.36 ± 1.36
MS + Activated charcoal (100)	92.69	24.28 ± 1.51	5.75 ± 0.17
MS + Activated charcoal (200)	89.30	24.16 ± 1.94	5.64 ± 0.35
F-value[2]	*	*	*
CD at 5% level	2.85	1.67	3.15

[1] Data indicate mean ± standard deviation. Ten replicates were used per treatments and experiment was repeated thrice.

[2] *: Significant at 5% level.

liferation from callus. Jose and Sateeshkumar (2004) accomplished a maximum mean of 10.4 shoots per shoot explants of *O. mungos* cultured on MS media supplemented with 2.22 µM BA. However, the present study clearly indicates that BA and KN in combination (0.25 mg L^{-1}) is a better choice for *O. mungos* as it significantly demonstrated the best morphogenetic response.

Shoot elongation

Though MS media with BA and KN in combination promoted multiple shoot formation, the regenerated shoots failed to elongate on the same media. This may be because of formation of shoot cultures in clumps. From the previous reports, it is evident that use of BA stimulates shoot proliferation while inhibiting shoot elongation (Figueiredo 2001; Purkayastha et al. 2008). Transferring cultures to media without growth hormones also did not support the shoot elongation. So developing a suitable media can promote proliferation and elongation of shoot clumps. To achieve shoot elongation, we used GA_3 at 0.25 – 2.0 mg L^{-1} in MS medium. The use of GA_3 at 1.0 mg L^{-1} stimulated the shoot elongation by 2.33 fold within 3 weeks (Fig. 1C). However in control treatments, the shoot elongation was completely absent (Table 2). The use of 1.0 mg L^{-1} GA_3 produced a mean shoot length of 5.1 ± 1.35. However, the stimulatory effect was less significant when GA_3 concentration was increased. The stimulatory effects of GA_3 to promote shoot elongation have been reported in several plant species (Paul et al. 2010; Purkayastha et al. 2008; Sugla et al. 2007). The explants continuously produced shoots during successive subculture on MS medium supplemented with 0.25 mg L^{-1} BA and KN.

In vitro rooting of plantlets and acclimatization

The effect of various factors such as strength of media, activated charcoal and types and concentration of auxin on rhizogenesis of *O. mungos* were evaluated (Table 3). Among the treatments tried, ½ strength MS medium was enough to get better rooting. The use of ½ strength MS medium for rooting has been reported in *O. prostrata* by Beegum et al. (2007). However, a high percent frequency of rooting (92.13%) was achieved within 15 days of shoot implantation on ½ strength MS media fortified with 100 mg L^{-1} activated charcoal. Mean number of roots/shoot (28.21 ± 1.14) and root length (6.30 ± 1.74 cm) was found to be superior on the same treatment (Fig. 1D). Among hormones, IAA was more effective than NAA in inducing roots. The highest frequency of root formation (70%), maximum number of roots (21.66 ± 0.51) and root length (5.67 ± 1.67 cm) were achieved on half-strength MS medium supplemented with 1.0 mg L^{-1} IAA (Table 3). Similarly in *O. mungos*, Jose and Sateeshkumar (2004) observed root formation in basal as well as in medium supplemented with auxins (12.3 µM, IBA and 1.07 µM, NAA). Activated charcoal is an anti-oxidant and known to induce rhizogenesis in *Maerua oblongifolia* (Rathore and Shekhawath 2011) and *Bauhinia cheilantha* (Gutiérrez et al. 2011). The encouraging effects of activated charcoal may be endorsed due to the creation of darker environment and

Table 3. Number of amplification products generated with the use of RAPD primers for the genetic analysis of *O. mungos*

S. No	Primer	Sequence	No. of bands
1	OPA 03	AGTCAGCCAC	4
2	OPC 11	AAAGCTGCGG	5
3	OPC 12	TGTCATCCCC	3
4	OPU 04	ACCTTCGGAC	3
5	OPU 08	GGCGAAGGTT	2

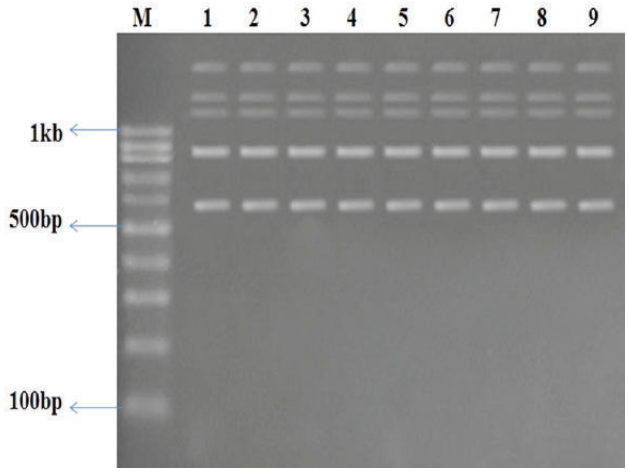

Fig. 2. Amplification pattern of mother plant, 2 - 9 showing amplification pattern of randomly selected *in vitro*-raised plantlets using random primer, OPC11. M 100 bp DNA ladder.

adsorption of undesirable/inhibitory substances (Thomas 2008). The result obtained by using half-strength MS medium containing activated charcoal was found to be superior when compared to the results obtained by using auxins. Though auxins (IAA and NAA) were shown to induce rooting with varying degrees, facilitated callus formation in a small number of shoots. This suggests that although the addition of auxins is beneficial for rooting, their use is not essential in *O. mungos*. Well developed plantlets having four to six fully expanded leaves and roots were acclimatized successfully in pots containing soil, sand, and manure (1 : 1 : 1) within 2 weeks. The plantlets were soon after established in a nursery with a survival rate of 95%. The established plants were morphologically similar with no visible variation (Figs. 1E, F).

RAPD analysis of regenerated plants

Micropropagation is used to obtain uniform planting material. However, it is necessary to authenticate the clonal fidelity of *in vitro*-regenerated plants to confirm the reliability on the protocol for mass multiplication. Since RAPD technique is being simple and cost effective, it has been widely used by many researchers to assess genetic stability in micropropagated plants (Agnihotri et al. 2009; Chalageri and Babu 2012; Chaudhuri et al. 2007; Paul et al. 2010; Sun et al. 2009). Hence, in our study we employed RAPD analysis to check the genetic uniformity among the micropropagated plants and mother plant. Out of 10 RAPD primers used, only four primers produced scoreable and reproducible bands. A total of 15 scoreable bands were obtained with an average of five per primer (Table 4). The primers OPC 11 and OPU 4 produced a maximum number of scoreable bands (Fig. 2). The results of the RAPD analysis showed no evidence of polymorphisms or changes between the micropropagated plants and mother plants of *O. mungos* suggesting the genetic stability among the plants.

HPLC analysis of campothecin content

The genetic control of biochemical character is also very important to guarantee stability in the drug yield, for the commercial production of elite *O. mungos* plant by tissue culture. So evaluation of the drug, camptothecin yielding potential of *in vitro*-generated plants and mother plant was assessed through HPLC analysis. Our results showed homogeneity in the chemical profile and campothecin content of the mother plant and the randomly selected micropropagated clones of *O. mungos* (Fig. 3). The level of camptothecin content from tissue culture raised clones of *O. mungos* was comparable in all the clones (0.0438 ± 0.18%, n = 03) and to that of the mother plant (0.043 ± 0.16%, n = 3).

Conclusion

The present study describes an efficient protocol for direct shoot regeneration of *O. mungos*. The genetic uniformity of micropropagated plants as assessed by using RAPD analysis confirms no genetic variations in the plants developed through an *in vitro* multiplication system. Also, the camptothecin content of micropropagated plants was similar to that of the mother plants. The *in vitro*-raised cultures could be used as a source for obtaining bioactive compound (camptothecin) on a large scale. Hence, this reliable plant regeneration protocol is not only useful for commercial multiplication but also for conservation of elite clones of *O. mungos* and other genetic manipulation experiments.

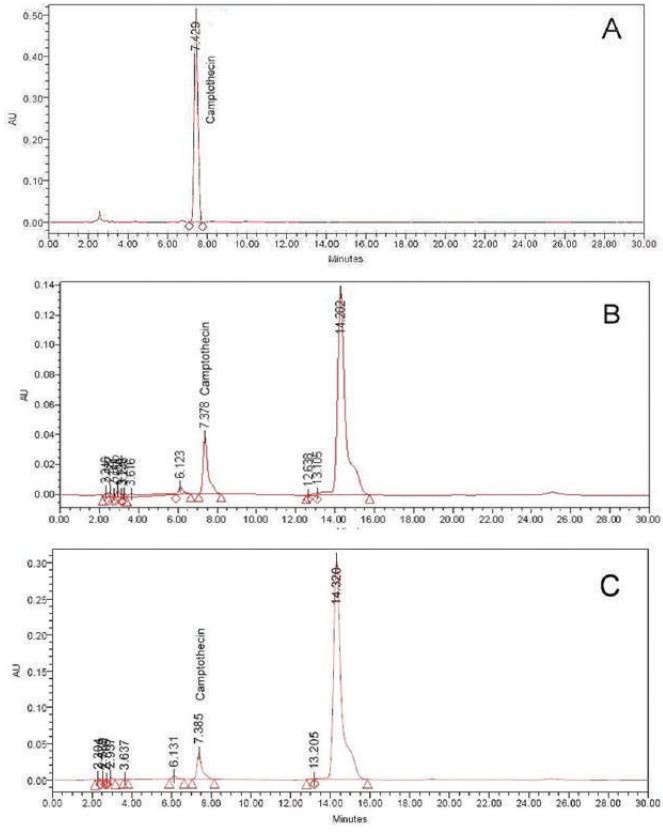

Fig. 3. (A) Standard of camptothecin. (B) HPLC analysis of camptothecin present in mother plant and (C) randomly selected micropropagated plants of *Ophiorrhiza mungos*.

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